Gene Therapy (2001) 8, 746–759

www.nature.com/gt

RESEARCH ARTICLE

Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity

J Nemunaitis[1,2], C Cunningham[1,2], A Buchanan[1], A Blackburn[1], G Edelman[1], P Maples[1], G Netto[2], A Tong[2], B Randlev[3], S Olson[4] and D Kirn[5]

[1]*US Oncology, Dallas, TX;* [2]*Sammons Cancer Center, Baylor University Medical Center, Dallas, TX;* [3]*Onyx Pharmaceuticals, Richmond, CA;* [4]*Warner-Lambert Corporation, Ann Arbor, MI, USA; and* [5]*Imperial Cancer Research Fund and Imperial College School of Medicine, London, UK*

Although genetically engineered adenoviruses hold promise for the treatment of cancer, clinical trial reports have utilized intratumoral injection to date. To determine the feasibility of intravenous delivery of ONYX-015, an E1B-55kD gene-deleted replication selective adenovirus with demonstrated clinical safety and antitumoral activity following intratumoral injection, we performed a clinical trial in patients with metastatic solid tumors. ONYX-015 was infused intravenously at escalating doses of 2×10^{10} *to* 2×10^{13} *particles via weekly infusion within 21-day cycles in 10 patients with advanced carcinoma metastatic to the lung. No dose-limiting toxicity was identified. Mild to moderate fever, rigors and a dose-dependent transient transaminitis were the most common adverse events. Neutralizing antibody titers significantly increased within 3 weeks in all patients. IL-6, γ-IFN, TNF-α and IL-10 increased within 24 h following treatment. Evidence of viral replication was detectable in three of four patients receiving ONYX-015 at doses* $\geq 2 \times 10^{12}$ *particles and intratumoral replication was confirmed in one patient. In conclusion, intravenous infusion of ONYX-015 was well tolerated at doses up to* 2×10^{13} *particles and infection of metastatic pulmonary sites with subsequent intratumoral viral replication was seen. The intravenous administration of genetically altered adenovirus is a feasible approach.* Gene Therapy (2001) **8,** 746–759.

Keywords: *ONYX-015; adenovirus; replication*

Introduction

Despite the successes of cytotoxic chemotherapeutics, new anticancer agents with improved safety and efficacy are still needed. Adenoviruses were initially engineered as replication-incompetent vectors whose only role was to deliver exogenous therapeutic genes.[1,2] A contrasting strategy has now emerged whereby replication-selective viruses, including adenoviruses, are themselves the therapeutic agent.[3–6] Proof of principle for this concept comes from animal studies where such viruses are found to replicate directly in cancer cells and destroy them, without the need for exogenous genes.[3–6] Eventually, superior efficacy may be achieved by combining these two strategies and arming replication-selective viruses with therapeutic transgenes.[7] However, to date, published clinical trials have been limited to intratumoral or intracavitary injections, and no data are as yet available on the safety or feasibility of intravenous adenovirus delivery to distant tumor metastases. Indeed, the recent death of a patient being treated with an adenoviral delivery vector for ornithine transcarbamylase deficiency, has raised concerns about the feasibility and safety of intravenous adenovirus administration.[8] Here we detail, for the first time, the initial results of a dose-escalation trial using the intravenous delivery of a replication-selective adenovirus in the treatment of cancer and showing it to be both well tolerated and feasible.

ONYX-015 is a replication-selective adenovirus with a deletion in the E1B-55kD gene region. Since the 55kD protein is necessary for inhibition of wild-type p53 function, as are similar proteins of other DNA viruses,[9,10] deletion of this gene should prevent efficient viral replication in cells with normal p53 function,[11] but allow replication in tumor cells harboring a p53 gene mutation or otherwise lacking functional p53. The p53 protein functions as part of a DNA repair mechanism in addition to its growth arrest and apoptosis induction functions, so that its loss plays a major role in tumorigenesis.[12] P53 also serves to protect the normal cell from viral replication if the cells should become infected by any of a number of viruses.[9–11] Sixty percent of human solid tumors contain mutations of the p53 gene, and many of the tumor cells with wild-type p53 gene sequences will have other abnormalities at the protein level.[13] Preclinical data, in general, confirm the modulation of ONYX-015 replication and oncolysis in tumor cell lines, although results have varied based on the cell system and endpoints studied.[11,14–18] Selection in clinical trials has been defined.

Correspondence: J Nemunaitis, US Oncology, 3535 Worth Street, Collins Bldg, 5th Floor, Dallas, Texas 75246, USA
Received 22 August 2000; accepted 22 December 2000

Phase I and II investigations in refractory head and neck cancer patients treated by intratumoral injection of ONYX-015 further support the selective nature of ONYX-015 replication in p53 mutant tumor tissue (58% necrosis rate for p53 mutant tumors *versus* 0% in tumors with normal p53 genome; $P = 0.017$) (Ganly *et al*, unpublished data). Adverse effects in normal tissue were not demonstrated despite injection of normal peritumoral tissue. A potentially synergistic interaction between chemotherapy and replication-selective adenovirus has also been discovered.[13,17,19,20] Chemotherapy-naive head and neck cancer patients treated with combination cisplatin, 5-fluorouracil (5-FU) and ONYX-015 had a response rate of 63% and minimal toxicity.[20] This compares favorably with the historical response rate of 35% for a similar population of patients. In light of these results, a phase III randomized trial is currently underway.

Intratumoral injection therapy, however, is limited to needle-accessible disease sites, whereas intravenous administration allows the potential for viral infection of even non-injectable disease sites. Intravenous administration of ONYX-015 to athymic mice bearing subcutaneous tumor xenografts supported the feasibility of this approach in mice.[18] Approximately 90% of ONYX-015 was recovered from the liver 3–6 h after a single infusion of 2×10^{10} particles; the liver had over 250 times more infectious virus particles per gram of tissue than tumor masses in the same mice. However, following tumor-selective replication for 3 days, no infectious virus was recovered from liver while titers had increased 100-fold in tumor tissue. *In situ* hybridization of the tumor tissue also showed focal areas of ONYX-015 replication within tumor margins and adjacent to feeding blood vessels. These results, therefore, documented the feasibility of adenoviral delivery to distant tumor masses following intravenous administration and established an LD_{50} in mice of $\sim 2 \times 10^{10}$ particles.[18] This dose is equivalent to 2×10^{13} particles in humans based on allometric scaling on a particle/g comparison of mice and human livers. Significantly, no additional toxicity was observed in animal models when i.v. ONYX-015 was combined with chemotherapy (5-FU, cisplatin, taxol).[13,19]

Although these preclinical mouse model results are encouraging, several problems exist with this model for use with replication-selective adenoviruses. First, normal murine tissues are not fully permissive for adenoviral replication, and therefore the rapid clearance of ONYX-015 from mouse liver may not occur in humans. Second, T cell-dependent immune responses are absent in athymic mice; the role of T cell-dependent humoral, cellular and cytokine-mediated immunity is therefore unclear. The intact immune response that adenovirus will encounter in patients may lead to more rapid clearance and/or inflammation-related toxicities. Finally, safety concerns have been raised following fatal hematologic and hepatic complications in a patient receiving an adenoviral gene delivery vector (replication-incompetent) via the bloodstream.[8]

To address these issues, we initiated a phase I dose-escalation trial to determine safety, pharmacokinetics, and feasibility of tumor uptake and replication of ONYX-015 administered intravenously alone, as well as in combination with carboplatin and paclitaxel, in patients with end-stage refractory carcinoma metastatic to the lung.

Results

Patient profile

The age, sex, Karnofsky performance status, site of disease, histologic tumor type, prior treatment and p53 mutation status of the 10 treated patients are shown in Table 1. The 10 patients described in this article received 126 intravenous infusions (42 cycles) of ONYX-015 (Table 2). Nine patients were entered into protocol, and one patient (C) was treated in nearly identical fashion on a compassionate basis following FDA and IRB approval (the patient had previously participated in a trial in which intratumoral injection of ONYX-015 virus at a dose of 2×10^{11} particles was administered for an initial 5 consecutive days of a 28-day cycle).[20] All patients were treated at the Mary Crowley Medical Research Center, Sammons Cancer Center, Baylor University (Dallas, TX, USA) from 11 January 1999 to 13 December 1999.

Adverse events

Three patients had grade 3 toxic events requiring hospitalization. All were classified as serious adverse events. None were related to the study agent. Grade 3 and 4 toxicity identified in patient C was related to tumor progression (dehydration and left carotid artery body compression related to tumor mass effect induced periodic hypotensive episodes). Grade 3 toxicity in patient 0003 was due to gram-negative sepsis from an infected indwelling catheter, and grade 3 toxicity in patient 0009 was due to a gram-positive intravenous central catheter infection. Neither of the bacterial infection events were related to study medication and completely resolved following antibiotics. Grades 2–4 clinical adverse events and relationship to study drug are shown in Table 3. Additional grade 3 toxic events not requiring hospitalization and not related to study medication occurred in patients 0004 and 0008 (Table 3).

The most common toxicities related to the administration of ONYX-015 were fever and rigors. All patients developed fever during either cycle 1 or 2 of ONYX-015. Fever most commonly developed within 4–8 h after infusion of ONYX-015 in cycle 1. Patients who received $\geq 2 \times 10^{12}$ particles of ONYX-015 ($n = 5$) also developed transient mild elevations (1.5–4.0 times the upper limit of normal) in AST and/or ALT during cycle 1. Patient 0007, who received 2×10^{13} particles, had transient ALT elevation approximately four times the upper limit of normal in cycle 1. Patients receiving less than 2×10^{12} particles in cycle 1 did not show any elevation of ALT or AST related to study agent, although a nonspecific elevation correlating with progression of malignancy within the liver was observed ($n = 2$ patients). There was no evidence of progressive clinical hepatotoxicity in any patient. Similarly, a transient elevation in circulating lymphocytes was observed in nine of 10 patients during cycles 1 and 2, but returned to baseline with continued ONYX-015 infusion (data not shown).

Pharmacokinetics

Evidence of viral genome could be detected for a minimum of 90 min after the end of ONYX-015 infusion in nine of nine patients at the initiation of the first and second cycle (ie fourth infusion) of therapy. One of four patients who received ONYX-015 at $\leq 6 \times 10^{11}$ particles had detectable viral genome at 6 h after infusion (patient

Table 1 Demographics

Patient No.	*Patient age*	*Sex*	*Karnofksy performance status (%)*	*Site of disease*	*Histologic tumor type*	*Prior treatment*	*P53 mutation*
C	46	M	90	Tongue, lung, pleura, sternum, T12 vertebra	Head and neck squamous cell carcinoma	Surgery (modified neck dissection) Radiation ONYX-015, cisplatin, 5-Fu	Point mutation exon 4, codon 63
0001	38	M	80	Diffuse to all organs	Carcinoma of unknown origin	Radical retropubic prostatectomy Radiation PEB (cisplatin, etoposide, bleomycin) Ifosfamide, adriamycin vincristine Taxotere/carboplatin, thalidomide Gemzar/taxol Doxil	Point mutation exon 9, codon 312
0002	71	M	80	Base of tongue, lung	Squamous carcinoma of head and neck	Radiotherapy Taxol, ifosfamide, cisplatin Carboplatin Taxotere, gemzar	Point mutation exon 4, codon 91
0003	48	F	100	Adrenal gland bed, kidney, lung	Adeno-carcinoma of adrenal gland	Bilateral adrenalectomy Left nephrectomy Radiation Mitotane Gemzar	Point mutation exon 4, codon 134
0004	58	M	90	Colon, liver, lung	Adeno-carcinoma of colon	Right and left hemicolectomy 5-FU/leucovorin	Point mutation exon 5, codon 132
0005	47	M	90	Lung	Adeno-carcinoma of lung	Taxol/carboplatin	Intron mutation of 12 base pairs, exon 9
0006	60	M	90	Thyroid, lung	Papillary carcinoma of thyroid	Partial thryoidectomy I-131	Intron mutation exon 8
0007	64	M	90	Colon, lung	Mucinous adeno-carcinoma of colon	Sigmoid colon resection Radiation 5-FU/leucovorin CPT-11	Point mutation exon 5, codon 175
0008	66	F	90	Lung	Adeno-carcinoma of lung	Carboplatin/taxol[a] Gemzar	Point mutations exons 2, 3, 5, codons 6, 32, 156, 158, 171, 174
0009	32	M	90	Sarcoma, lung	Osteosarcoma	BCNU/temozolomide LY231514/irinotecan CPT-11 Ifosfamide, adriamycin, DTIC, tazarotene	Intron mutation of 7 base pairs, exon 11

[a]Patient 0008 had previous breast cancer in 1991 for which she underwent a radical mastectomy followed by adjuvant therapy with adriamycin, cytoxan and tamoxifen.

0001, 2.11×10^5 particles/ml) in cycle 1, whereas all five patients receiving $\geq 2 \times 10^{12}$ particles had minimally detectable ONYX-015 at 6 h after the end of infusion (patient 0005, 17.3×10^5; patient 0006, 19.9×10^5; patient 0007, 14.7×10^5; patient 0008, 2.2×10^5; patient 0009, 2.5×10^5 particles/ml). All patients, except 0001, had detectable ONYX-015 genome at 6 h after the end of ONYX-015 infusion at initiation of cycle 2.

Plasma titers of viral DNA were independent of dose over the initial dose range of 2×10^{10} to 6×10^{11} particles. At 2×10^{12} particles and above, there appeared to be a greater plasma PCR signal, but it was not statistically significant (Figure 1). DNA titers declined with a half-life of about 20 min over the first 2 h following dosing (Figure 2), then did not change as a function of time from 2 to 6 h after treatment. Higher doses yielded higher levels within this time-window. There were no observable differences between cycle 1 and cycle 2 in dose or time dependencies or in individual systemic exposure to the ONYX-015 genome following equivalent single doses.

Table 2 Treatment summary

Patient No.	*ONYX-015 dose (particles)*	*No. infusions*	*No. cycles on study*	*No. chemotherapy infusions*[d]	*Chemotherapy dose reduction/reason*	*Total No. chemo cycles*
C	2×10^{10}[a]	1	7	C3D1–C6D1	Full dose	5
	2×10^{11}[a]	1		C6D8–C7D15	30% decrease due to neutropenia	
	2×10^{12}[a]	19				
0001	2×10^{10}	12	4	0	No chemotherapy; baseline thrombocytopenia persistence	0
0002	6×10^{10}	6	2	0	No chemotherapy per patient decision	0
0003	2×10^{11}	15	5	C3D1–C3D15	Full dose	3
				C4D1–C5D15	Carbo reduced by 10%	
0004	6×10^{11}	12	4	C3D1–C3D15	Full dose	2
					C4D1 not given due to grade 3 diarrhea	
				C4D8 and D15	50% dose reduction	
0005	2×10^{12}	24	8	C3D1–C8D15	Full dose	6
0006	6×10^{12}[b]	1	4	C3D1 and D8	Full dose	2
	2×10^{12}[b]	11		C3D15	40% dose reduction in taxol due to grade 3 neutropenia	
				C4D1	Full dose	
				C4D8 and D15	40% dose reduction due to neutropenia	
0007	2×10^{13}[c]	1	4	C3D1 and D8	Full dose	2
	2×10^{12}[c]	11		C3D15	25% increase in taxol and 50% decrease in carbo due to low counts	
				C4D1–C4D15	50% decrease in taxol and 50% increase in carbo	
0008	2×10^{12}	6	2	C1D7–C2D15	Full dose	2[d]
0009	2×10^{12}	6	2	C1D7–C2D15	Full dose held 3 weeks due to line infection	2[d]

[a]Patient C received a dose of 2×10^{10} particles on day 1 of cycle 1 and 2×10^{11} on day 8 of cycle 1. All subsequent infusions were 1×10^{12} particles.
[b] Patient 0006 received a dose of 6×10^{12} particles on day 1 cycle 1 and 2×10^{12} on all subsequent infusions.
[c]Patient 0007 received a dose of 2×10^{13} particles on day 1 cycle 1 and 2×10^{12} on all subsequent infusions based on protocol design.
[d]Chemotherapy (taxol at 80 mg/m^2 i.v. qd/wk; carboplatin ANC of 2 i.v. qd/wk) was initiated on day 7 of cycle 1 in patients 0008 and 0009.

Evidence for viral replication

Detection of ONYX-015 genome in plasma by quantitative PCR on day 7 after the end of infusion is summarized in Table 4. Day 7 plasma sample positivity was more common at doses $\geq 2 \times 10^{12}$ (13/27, 48% *versus* 7/21, 33%) particles. Furthermore, persistent viral genome at day 2 and day 7 after a virus infusion was shown in 18 of 34 (53%) patient samples receiving $\geq 2 \times 10^{12}$ particles compared with seven of 24 (29%) patient samples at the dose of $\leq 6 \times 10^{11}$ particles. Overall, 46% (17 of 37) of all plasma samples had detectable ONYX-015 genome in cycle 1, and 38% (eight of 21) of plasma samples were positive in cycle 2.

In order to confirm that replication was occurring at doses $\geq 2 \times 10^{12}$ particles, viral genome concentration from blood drawn 48 h after infusion of virus was compared to the 6-h viral genome blood concentration. Three of four patients with samples drawn had an increase in viral concentration (2.5 to 10-fold) between 6 and 48 h (one patient missed the blood draw). Viral genome increased from 4.7×10^6 to 8.6×10^6 genomes/ml in patient 0007, from 2.2×10^5 to 8.9×10^6 genomes/ml in patient 0008, and from 2.5×10^5 to 1.7×10^6 genomes/ml in patient 0009.

Once viral replication had been documented, attempts were made to confirm that viral replication was occurring within the tumor tissue as predicted. Because tumor tissue was non-evaluable in most cases (due to necrosis and/or inaccessibility), only one tumor biopsy sample was evaluable for immunohistochemical staining for replication and/or for ONYX-015-specific PCR (to confirm the virus identity). Immunohistochemical staining of adenoviral capsid antigen confirmed intratumoral replication (Figure 3), and quantitative PCR testing (Figure 4) confirmed that this was ONYX-015 (ie not wild-type adenovirus) at a concentration of 5.91×10^4 ONYX-015 genomes/μg of DNA analyzed. This patient (0008) had multiple positive day 7 plasma PCR samples and was one of the three patients in whom quantitative PCR indicated increasing plasma genome levels at 48 h and day 7 in comparison to the 6-h plasma genome level. Some capsid antigen expression can also be identified in adjacent bronchial alveolar cells; this appears to have been due to phagocytosis of adenoviral protein by adjacent pneumocytes. Surrounding pulmonary tissue did not show immunohistochemical evidence of adenovirus replication or histologic evidence of toxicity. Patient C underwent autopsy analysis 134 days following his last ONYX-015 infusion. No evidence of ONYX-015 replication was identified in normal organ tissues or malignant tissue by quantitative PCR.

The role of neutralizing antibodies in blocking infec-

Table 3 Grade 2–4 adverse events

Patient No.	*Toxic event*	*Grade*	*Related to study drug*
C	Hypotension	4	No
	Brachycardia	4	No
	Dehydration	3	No
	Fatigue	3	No
	Candida – mouth	2	No
	Hot and cold flushes	2	Yes
	Nasal congestion	2	No
	Constipation	2	No
	Dizziness	2	Possibly
	Dysphagia	2	Possibly
	Diarrhea	2	Possibly
	Chills	2	Yes
	Fever	2	Yes
	Anxiety		No
0001	Dyspnea with exertion	2	No
	Tumor pain	2	Yes
	Incisional (by site) pain	2	No
	Swelling by site	2	No
	Chills	2	Yes
	Coughing blood	2	Yes
	Fever	2	Yes
	Cough	2	No
0002	Fatigue	2	Yes
	Fever	2	Yes
	Non-productive cough	2	Yes
0003	Right PAC (i.v.) site redness	2	No
	Fatigue	2	No
	Fever	2	Yes
	Increased creatinine	3	No
	Sepsis	3	No
	Acne	2	No
0004	Right side liver/abdominal pain	3	No
	Diarrhea	3	No
	Constipation	2	No
	Fatigue	2	No
	Sleeplessness	2	No
	Pain around PAC (i.v.) incision site	2	No
	Fever	2	Yes
	Malaise	2	Yes
0005	Chills	2	Yes
	Wheezing	2	No
	Upper right lung pain	2	No
	Right lung base pleuritic pain	2	No
	Fever	2	Yes
	Right flank/abdominal pain	2	Yes
0006	Fever	2	Yes
	Chest discomfort	2	No
	Sleeplessness	2	No
	Nausea	2	Yes
	Cough	2	No
	Fatigue	2	Yes
	Body aches	2	Yes
0007	Nausea (intermittent)	2	Yes
	Vomiting	2	Yes
	Fever	2	Yes
	Chills	2	Yes
0008	Decreased WBC	3	No
	Increased cough	2	Yes
	Blood tinged sputum	2	Yes
	Increased shortness of breath	2	Yes
	Fever	2	Yes
	Fatigue	2	Yes
	Diarrhea	2	No
0009	Fever	3	Yes
	Catheter infection	3	No
	Muscle cramps	2	No
	Fatigue	2	Yes
	Chills	2	Yes
	Body aches	2	Yes
	Nausea and vomiting	2	No

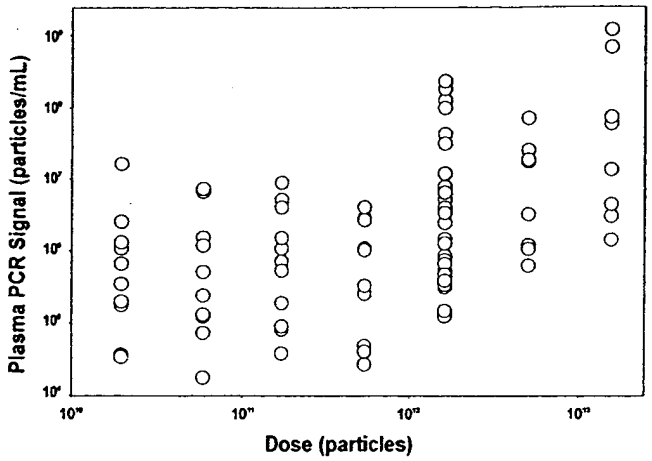

Figure 1 Plasma PCR signal versus dose of ONYX-015. Quantitative determination of ONYX-015 genome was done using a TaqMan based assay.

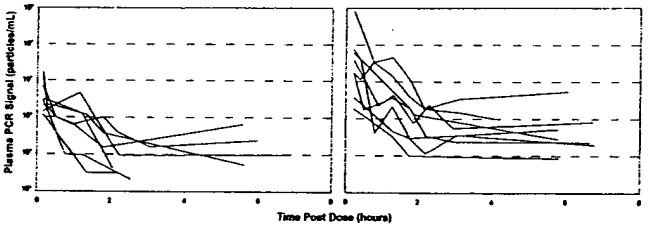

Figure 2 Plasma PCR signal versus time post-dose of ONYX-015. The left hand figure evaluates cycles 1 and 2 of patients receiving $\leq 2 \times 10^{11}$ particles; the right hand figure evaluates cycles 1 and 2 of patients receiving $\geq 2 \times 10^{12}$ particles.

tion could not be assessed. All high-dose patients were antibody negative at baseline.

Neutralizing antibody response

Two patients (Nos 0003 and C) had elevated neutralizing antibody titers at baseline (Table 5). Patient C had previously received ONYX-015 via intratumoral injection. All patients showed marked elevation in neutralizing antibody titers within 2–3 weeks of the first injection (Table 5).

Cytokine response

Cytokines IL-6, IL-1α, IL-1β, TNF-α, γ-IFN, IL-10 and IL-2 were assessed in patients who received ONYX-015 at

Table 4 Detectable ONYX-015 genome in plasma 7 days following i.v. infusion

Patient No.	*Cycle 1 (infusion No.) PCR results day 2, day 7 (×10⁴ genomes/ml)*			*Cycle 2 (infusion No.) PCR results day2, day 7 (×10⁴ genomes/ml)*		
	1	*2*	*3*	*1*	*2*	*3*
0001	LLD, LLD	ND, 3.4	ND, LLD	ND, LLD	ND, ND	ND, ND
0002	ND, LLD	ND, LLD	ND, LLD	ND, LLD	ND, LLD	ND, ND
0003	LLD, LLD	ND, 3.3	ND, 140	ND, 2.3	ND, 14	ND, ND
0004	LLD, LLD	ND, LLD	ND, LLD	ND, LLD	ND, 58	ND, ND
0005	ND, LLD	ND, LLD	ND, LLD	LLD, LLD	ND, LLD	ND, ND
0006	3.2, LLD	ND, LLD	ND, LLD	ND, LLD	ND, LLD	ND, ND
0007	860, 49	ND, 5.6	ND, LLD	ND, LLD	ND, LLD	ND, LLD
0008	890, 7.1	ND, 68	ND, 200	ND, 56	ND, 280	ND, 68
0009	170, 99	ND, 130	ND, 19	LLD, ND	ND, ND	ND, ND
C	ND, LLD	ND, 35	ND, 35	ND, 25	ND, 170	ND, ND

ND, not done; LLD, level lower than detectable.

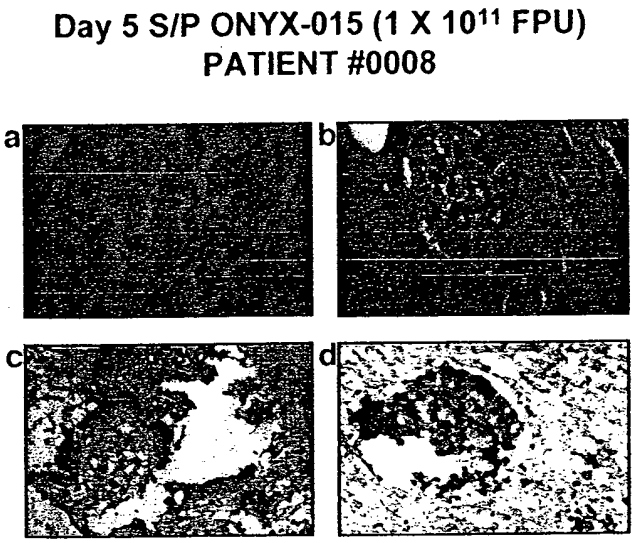

Figure 3 (a) Day 5 biopsy of tumor from the lung of patient No. 0008; positive control for adenovirus antigen MoAb. (b) Histochemistry of malignant tumor tissue of patient No. 0008. (c) Positive uptake of MoAb to capsid antigen in bronchial epithelial cells of patient No. 0008. (d) Positive uptake of MoAb to capsid antigen in malignant cells of patient No. 0008.

doses ≥2 × 10¹² particles (patients 0005–0009) (Figure 5a, b). Significant elevation (>25% of baseline value) of IL-6, IL-10, TNF-α, and γ-IFN, were observed in the majority of patients tested after infusion on days 1 to 3 of cycle 1 and days 1 to 3 of cycle 2. Patient 0006 was unavailable for cycle 2 due to inability to acquire the follow-up samples after cycle 2 baseline assessment. We did not detect any significant increase in IL-1α, IL-1β or IL-2 levels in patient serum collected throughout the course of study. However, an early rise with subsequent drop back to baseline within the first 24 h could have been missed; this pattern of rise and fall was demonstrated for IL-1 following intra-arterial administration of ONYX-015 on another trial (Kirn *et al*, unpublished data). Elevated levels of serum IL-6 from 128% to 834% over baseline (from 5.4 to 50.6 pg/ml; No. 0006) were observed in five of five patients within 24 h after infusion at day 1, cycle 1 and probed within 6 h of infusion. A similar increase of 28%

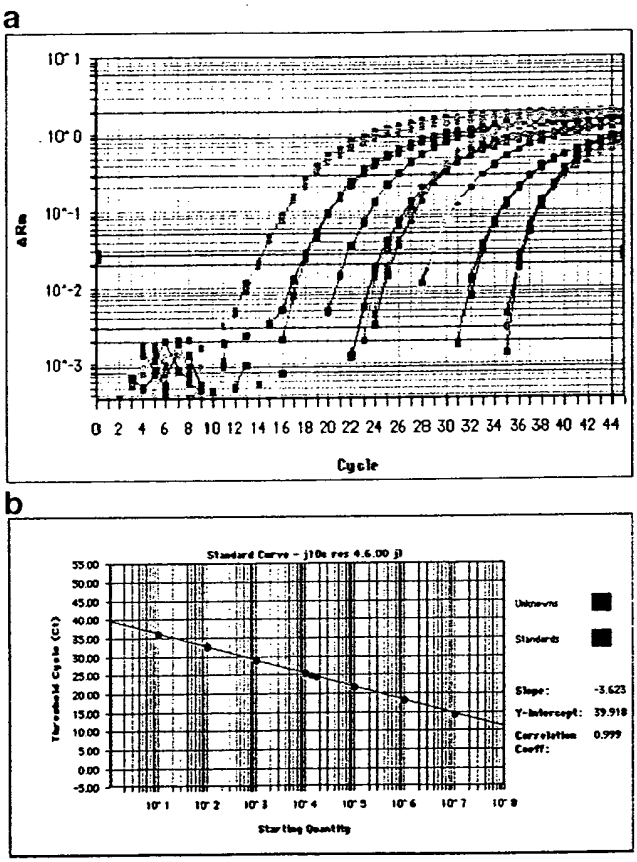

Figure 4 (a) Amplification plot of ONYX-015 DNA of patient 0008 tumor tissue in comparison to standard and negative controls. All samples and controls were run in duplicate. Patient samples were positive at cycle 22 and cycle 23 (red and blue curves at cycles 22 and 23). Fluorescent reporter units from the PCR reaction are shown at the Y axis and the number of PCR cycles is marked on the X axis. (b) Standard curve of known quantities of ONYX-015 DNA (2.61 × 10¹, 2.61 × 10², 2.6 × 10³, 2.61 × 10⁴, 2.61 × 10⁵, 2.61 × 10⁶ and 2.61 × 10⁷ genomes/μg) as a function of the cycle number (Y axis) at which significant detection occurred. Patient No. 0008 is shown in red.

Table 5 Neutralizing antibody titers

Patient No.	*Cycle*	*Day*	*Antibody titers*
C	1	1	>1280
C	1	8	>81920
C	1	15	>57353
C	2	1	>75491
C	2	8	>81920
C	2	15	>81920
0001	1	1	<20
0001	1	8	<20
0001	1	15	91
0001	2	1	378
0001	2	8	1464
0002	1	1	<20
0002	1	8	22
0002	1	15	1032
0002	2	1	1109
0002	2	8	1806
0002	2	15	5324
0003	1	1	967
0003	1	8	14184
0003	1	15	71730
0003	2	1	50186
0003	2	8	48041
0003	2	15	44213
0004	1	1	<20
0004	1	8	69
0004	1	15	385
0004	2	1	906
0004	2	8	2393
0004	2	15	3437
0005	1	1	<20
0005	1	8	1378
0005	1	15	3005
0005	2	1	14215
0005	2	8	11503
0005	2	15	12465
0006	1	1	<20
0006	1	8	141
0006	1	15	670
0006	2	1	4454
0006	2	8	1198
0006	2	15	843
0007	1	1	<20
0007	1	8	121
0007	1	15	190
0007	2	8	494
0007	2	15	1760
0008	1	1	<20
0008	1	8	5518
0008	1	15	6362
0009	1	1	27

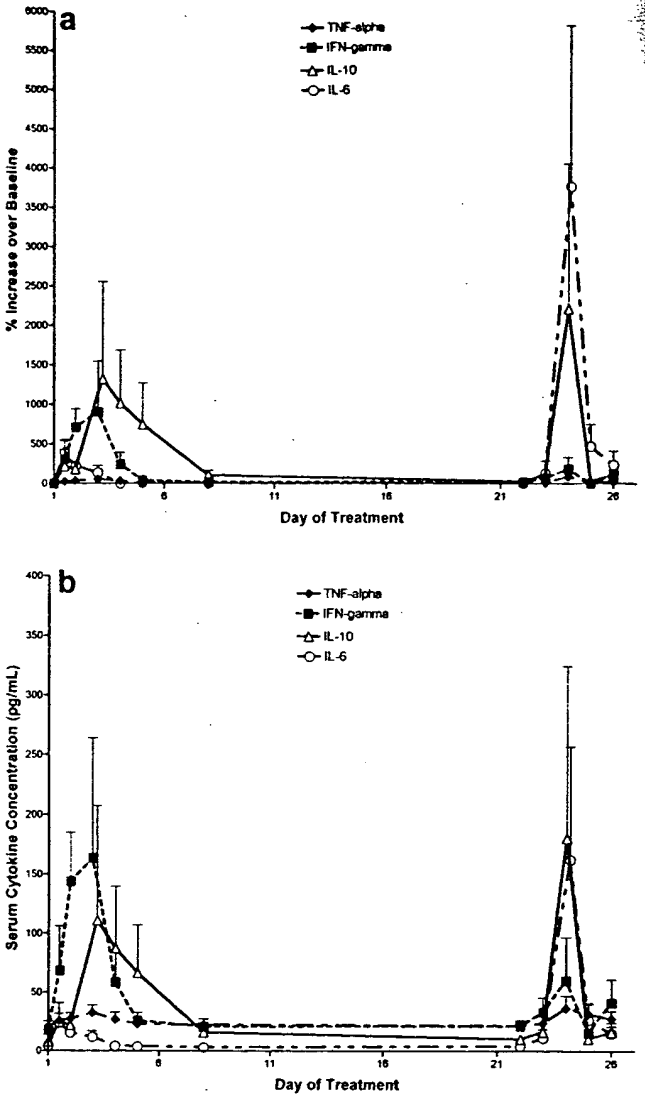

Figure 5 Percent increase over baseline (a) and absolute (pg/ml; b) of mean serum cytokine levels as determined by ELISA assay at initiation of cycle 1 and cycle 2 in patients treated with intravenous ONYX-015 ≥2 × 10^{12} particles (n = 5). Cycle 1 was administered on day 1. Cycle 2 was administered on day 22. Values represent mean ± s.e.m.

to 586% was seen in three of four patients (0005, 0007, 0009) ≤24 h at cycle 2. This increase subsided by days 3–4 and returned to baseline before the next infusion of ONYX-015, 7 days later in all patients. Concurrent or delayed increase in serum IL-10 (five of five patients tested; 70–6259% increase) and γ-IFN (four of five patients; 865–3321% increase) were observed, peaking at days 2 (γ-IFN) and 3–4 (IL-10) of cycle 1. Markedly higher increases of IL-6 and IL-10 but not γ-IFN were seen at cycle 2. There were relatively modest increases (26–170%; 22–42 pg/ml) of TNF-α in four of five patients at days 2–5 of treatment in cycle 1. Only one patient (0007) was found to increase TNF-α at cycle 2. Impressive increases were observed at the 6-h time-point only with IL-6 suggesting a possible correlation of IL-6 with initial fever commonly observed 4–8 h after administration of cycles 1 and 2. All but one instance was correlated with a >25% increase of IL-6. Patients 0005, 0006, 0007, 0008 and 0009 all had transient fever ≤10 h from initial infusion of ONYX-015.

Response

Tumor response is shown in Table 6. Eight patients had stable disease at the 2-month assessment, one a mixed response, and one progressive disease. Time to progression and survival is also shown in Table 6. Although no objective responses were demonstrated with ONYX-015 alone in this safety study, tumor stabilization of 6.5+ months was demonstrated in two patients with ONYX-015 and carboplatin/paclitaxel. One (No. 0005) of the two patients had previously failed prior carboplatin/taxol treatment.

Table 6 Response

Patient No.	*Optimal response*	*Time to progression (days)*	*Survival (days)*
C	MR[a]	148	281
0001	SD	107	197
0002	SD	41[b]	298
0003	SD	103[c]	312
0004	SD	67	218
0005[d]	SD	192+	449+
0006	SD	294	349+
0007	SD	91	300
0008	PD	77	223+
0009	SD	82+	162

[a]Mixed response resolution of T12 lesion and pleural mass regression following radiation in combination with taxol, irradiation, and ONYX-015 at cycle 6. Overall disease not reduced 50%.
[b]Out of state patient unable to remain in Dallas after cycle 2.
[c]Patient taken off study due to renal failure.
[d]Patient failed prior chemotherapy with carboplatin and taxol.

Discussion

We demonstrate, for the first time, that an attenuated adenovirus can be administered safely as a single intravenous infusion to a dose of 2×10^{13} particles and as weekly infusions at doses up to 2×10^{12} particles. No dose-limiting toxicities were identified; mild to moderate fever, rigors and mild transient transaminitis (following high doses only) were the most common toxicities. This lack of significant toxicity is remarkable given evidence for ongoing viral replication and shedding into the circulation for several weeks in high-dose patients. Toxicity was not altered when ONYX-015 was administered in combination with carboplatin and taxol. These results suggest that future therapeutic designs can safely incorporate administration of intravenous ONYX-015 in humans. Importantly, viral infection of metastatic tumor deposits was documented at these well-tolerated doses of $\geq 2 \times 10^{12}$ particles. Both acute inflammatory cytokine-mediated immunity (primarily IL-6, IL-10, interferon-gamma, tumor necrosis factor) and humoral immunity were induced rapidly following intravenous administration. A correlation between IL-6 induction and the initial febrile response was suggested. This is consistent with toxicity attributed to IL-6 during preclinical and clinical investigation of recombinant human (rh)IL-6 protein,[21,22] and observations in other trials involving adenoviral vectors.[8] Up-regulation of IL-6, which we observed, is consistent with an acute phase reaction that peaked within 6 h of initial viral infusion and first of the host's triphasic immune response.[23] Elevated γ-IFN levels that peaked at 24–48 h after infusion are indicative of NK, and subsequently TH1-mediated immune activation events. Maximal IL-10 levels at 72–96 h after infusion may reflect collateral TH2 activation, although IL-4 and IL-5 determinations are needed to confirm this hypothesis. Similarly, others have shown adenoviral antigen stimulation of TH1 and TH2 response.[24–26] Our findings with a limited number of patients also suggest preferential TH2 activation at the time of viral reinfusion at cycle 2, based on the marked increase in IL-2 and IL-10 within 72 h.

Our results demonstrate that infection of, and replication within, tumors by ONYX-015 is feasible following intravenous administration. More than half of the plasma samples from patients receiving doses greater than 2×10^{12} particles showed persistence of viral genome in blood more than 24 h after administration (nearly half at 7 days). Without replication, the viral genome is not observed in circulation for more than 24 h in animal models (and usually not more than 6 h) after infusion. Interestingly, the levels of viral genome detected in the plasma of three patients 48 h after infusion were 2.5- to 10-fold higher than levels in the plasma at 6 h. Therefore, significant on-going replication was taking place. Immunohistochemistry and quantitative PCR analysis of tumor tissue was used to confirm intratumoral viral in the one patient with an accessible and evaluable tumor. We cannot rule out viral replication at sites outside the tumor, as well, but even if this did occur, it was not associated with any clinically significant toxicity.

Although it is possible that antibody binding will decrease the efficiency of tumor injection, viral pharmacokinetics were not demonstrably altered by antibody formation. Future studies will need to address this issue. If necessary, approaches to block antibody development can be tested.

Although primarily a safety study, we did confirm intratumoral delivery and replication in at least one patient using this systemic approach. If intravenous delivery of virus is to evolve into an effective therapy, consistent delivery to, and uptake by, the tumor will be necessary. Several approaches may be taken to improve the efficacy of future intravenous treatments. One is to combine the favorable attributes of replication-selective viral therapy with gene delivery;[6,7] replication-selective viruses can be engineered to express therapeutic genes (eg cytokines, prodrug-activating enzymes) and thereby improve tumor destruction as compared with the virus alone. Inhibition of immune-mediated viral clearance may also be possible through the use of immunosuppressive regimens, particularly in light of the safety demonstrated on this trial. It is also clear that replication-selective adenoviruses are effective and potentially synergistic in combination with standard chemotherapeutics.[19,20,27] Since the mechanisms of action of adenoviral therapy and chemotherapy are very different, and overlapping toxicities are not apparent, this approach has great promise.

Future studies may test approaches to reduce hepatic clearance of therapeutic adenoviruses. Preclinical studies indicate that more than 80% of ONYX-015 and other adenoviruses are taken up by the liver following intravenous administration.[27] Thus, the delivery of adenovirus to metastatic tumor deposits, and the resulting antitumoral activity, should be improved if hepatic uptake is inhibited.[28–30] Furthermore, approaches which decrease hepatocyte and Kupffer cell uptake such as infusion of silica and/or multi-lamellar lyposomes (Cl_2MBP lyposomes),[31–44] have been shown to delay elimination of adenoviral vectors significantly. A second important factor influencing internalization of adenovirus and induction of actin cytoskeleton reorganization for endocytosis of the viral particle to take place is the expression of coxsackie-adenovirus receptor (CAR) on target cells which serves as a primary receptor for adenovirus.[45] Interaction with a second receptor, α_V-$\beta_{3,5}$ integrin, facilitates virus internalization into cells.[46] Internalization

probably occurs by clathrin-mediated endocytosis,[47,48] an actin-dependent process, under the control of the PI3K pathway,[49] and may be therapeutically modulated by blocking of the Rho family of small GTP-binding proteins that serve as regulators of PI3K activation.[45,50–53] Strategies to modulate the expression levels or function of these receptors in tumor tissues and/or normal organs are being developed and might eventually be tested in clinical trials.

Another major factor influencing the efficacy and toxicity of adenoviral agents is the immune response to adenovirus administration and replication.[29,46,54–60] Adenovirus-mediated gene transfection in immunodeficient mice[24,25,61–67] or immunocompetent animals treated with immunosuppressive agents[67–68] has resulted in prolonged transgene expression.[25,26,64,66,67,69–77] Future studies exploring intravenous infusion of ONYX-015 may involve testing of transient immunosuppression.

The clearance of adenovirus is also affected by the expression of immunomodulatory gene products from the virus itself. For example, the E1B 19kD gene is responsible for blocking *fas* and TNF receptor-induced apoptosis, whereas the adenovirus early transcription unit (E3) encodes for polypeptides (14.7k, 10.4, 14.5),[78–82] which function to block directly TNF-α activation as well as apoptotic pathways shared by TNF-α and *fas*.[78,80,83] The E3 gp-19k protein functions to bind and retain MHC class I molecules within the endoplasmic reticulum, thus preventing surface presentation of viral antigens, thereby limiting class I-restricted CTL clearance of virally infected cells.[79,82,84–87] The E3b region of ONYX-015 is deleted and this virus may therefore be hypersensitive to clearance by *fas* and/or TNF. Replacement of this gene region may, therefore, decrease viral clearance, increasing expression of viral genes that suppress immune recognition which may improve replication.[17,78,88]

In conclusion, results of this trial demonstrate that ONYX-015 can be safely administered to refractory cancer patients as a single agent or in combination with chemotherapy. These results open the door to innovative therapeutic approaches utilizing attenuated replication-selective adenoviruses as a new therapeutic modality via intravenous delivery in cancer patients with epithelial or even hematologic malignancy.[89] Methods of modulating adenoviral clearance, uptake, tropism for malignant cells and functional activity need to be further investigated. Future directions will include the use of replication-selective viruses in combination with standard oncologic treatment regimens, as well as the expression of exogenous therapeutic genes.[7,90]

Methods

Test article

ONYX-015 is a chimeric human group C adenovirus (Ad2 and Ad5), originally constructed in the laboratory of Arnold Berk, that does not express the 55kD product of the E1B gene.[91] The virus contains a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55kD protein. In addition, a C to T transition at position 2022 in E1B generates a stop codon at the third codon position of the protein. These alterations eliminate expression of the E1B 55kD gene in ONYX-015 infected cells. ONYX-015 was grown and titered on the human embryonic kidney cell line HEK293 as previously described.[13]

Study design

This was an open-label phase I, dose-escalation study. Patients received ONYX-015 on days 1, 8 and 15 of each 21-day cycle. Dosage cohorts were as follows (one patient at each dose level): (1) 2×10^{10} particles; (2) 6×10^{10} particles; (3) 2×10^{11} particles; (4) 6×10^{11} particles; (5) 2×10^{12} particles; and (6) 6×10^{12} particles for the first dose, then 2×10^{12} particles for subsequent doses; (7) 2×10^{13} particles for the first dose, then 2×10^{12} particles for subsequent doses; (8) 2×10^{12} particles with chemotherapy starting cycle 1, day 8. Carboplatin (AUC 2) and taxol (80 mg/m^2) were administered in combination with ONYX-015 starting with cycle 3 (continued for all subsequent cycles) in cohorts 1–7 and starting with cycle 1, day 8 in cohort 8. A single patient was treated on a compassionate use protocol testing intra-patient dose escalation as follows: 2×10^{10}; 6×10^{10}; 2×10^{11}; 6×10^{11}; 2×10^{12} particles. Treatment was continued as long as patients maintained a status of stable disease and did not develop significant toxicity.

ONYX-015 handling and processing

ONYX-015 is formulated as a sterile viral solution in TRIS buffer (10 mM TRIS pH 7.4, 1 mM $MgCl_2$, 150 mM CaCl, 10% glycerol). The solution is supplied frozen (−20°C) in single-use, plastic screw-cap vials. Each vial contains 0.5 ml of virus solution at a specified viral titer. Vialed virus solution was warmed and diluted to the appropriate titer for dosing. Following warming, ONYX-015 was maintained at 2° to 8°C during dilution and handling, except for warming to room temperature immediately before administration.

Drug preparation was performed within 10 min before tumor infusion, using plastic syringes. Vials of ONYX-015 were opened and the appropriate volume of virus diluted to 10 ml with normal saline in a biologic safety cabinet. All waste items were disposed of in biohazard containers and were autoclaved and incinerated. For dose levels of 3×10^{11} and 1×10^{12} only, the prepared dose of ONYX-015 was injected into a 100 cc bag of normal saline for infusion.

ONYX-015 intravenous infusion procedure

A single infusion of 10 ml of ONYX-015 with normal saline was administered over approximately 3–5 min, as specified for each cohort in the product information sheet through a peripheral or central intravenous line (22-gauge catheter). For dose levels of 6×10^{12} and 2×10^{13}, a 100 cc bag of normal saline with the prepared dose of ONYX-015 was administered via infusion pump over a 10-min period.

Dose-escalation scheme – cycle 1

One patient was entered per dose level until the highest dose was shown to be safe; at this point three additional patients were treated at the highest dose level. Patients were enrolled sequentially into treatment cohorts with at least a 2-week interval between each cohort. Dose escalation was based on the toxicity seen with the first cycle of therapy. If a DLT related to ONYX-015 was observed in a cohort related to ONYX-015, up to six patients were to be enrolled at that dose level. If a DLT, felt not to be

related to ONYX-015 occurred, that patient was replaced. Patients were enrolled until a second DLT occurred (which defined the toxic dose) or until six patients were enrolled at that dose level. The MTD was defined as the dose immediately below the dose at which two patients experienced a DLT after their first treatment with ONYX-015. DLT was defined as grade 4 toxicity of any duration and grade 3 toxicity lasting more than 5 days. A minimum of a 1-week interval between treatment of each of these patients was required unless toxicity was observed at >1 week in which case the duration between patients was a minimum of the duration of time at which the toxicity was observed from the treatment date.

Chemotherapy dosing

A combination of carboplatin (i.v. dose at AUC of 2 per standard practice) and paclitaxel (80 mg/m^2 i.v. per standard practice) was administered weekly concurrently with systemic virus administration. Pre-medications for chemotherapy were given per standard of practice for the clinic. They involve dexamethasone 20 mg/12 h then 2 h before dosing; diphenhydramine 25 mg and ranitadine 50 mg i.v.; then odensatron 1 mg and dexamethasone 10 mg i.v. just before dosing.

Study population

Patients with advanced metastatic cancer who failed one prior therapeutic regimen and for whom a curative therapy is not available were eligible. Patients were required to have histologically or cytologically confirmed carcinoma, tumor not curable with surgery or other standard modalities, and a documented p53 gene mutation. For dose levels greater than 6×10^{12} particles, patients were required to have disease involving the lung. Other eligibility criteria included a Karnofsky performance status of ≥90%, life expectancy of ≥3 months, age ≥18, absolute neutrophils >1500/μl, hemoglobin >9 mg/dl, platelets >75000/μl, creatinine <2.0 mg/dl, AST and ALT >3.0-fold upper limit of normal, PTT within normal limits, and total bilirubin <2.0 mg/dl, and consent for study participation on an IRB-approved informed consent form. Women and men of reproductive potential were required to use a reliable form of contraception.

Patients with the following were excluded from study: >50% liver replacement by tumor as determined radiographically; ongoing active infection (including human immunodeficiency virus); viral syndrome diagnosed within the last 2 weeks; chemotherapy within the last 3 weeks (6 weeks for nitrosourea or mitomycin-C); radiotherapy to tumor site within the last 4 weeks (if this is the only site of disease); treatment with any other investigation therapy within the last 4 weeks; concomitant hematological malignancy (ie chronic lymphocytic leukemia, non-Hodgkin's lymphoma); and current requirement for chronic immunosuppressive medication including glucocorticoid or cyclosporin, or chronic use of any such medication within the last 4 weeks. Pregnant or lactating females were excluded, and patients previously participating in research protocols involving administration is adenovirus vectors were excluded (with the exception of one compassionate use protocol patient).

Tumor assessment

Up to five masses were measured for response assessment. A complete response (CR) was defined as complete disappearance of all tumors at the assessed site, partial response (PR) was defined as regression of the tumor mass by ≥50% but <100% with no growth of any individual lesion, stable disease (SD) was defined as tumor decrease by <50% or increase in size by <25%, and progressive disease (PD) was defined as a ≥25% increase in tumor size. Tumor response was assessed by CT scan of relevant organs each odd cycle. CT confirmation of all PRs and CRs 4 weeks after initial documentation of the response was required.

Adverse event definition

Severity of adverse events were assigned according to the NCIC-CTG Expanded Common Toxicity Criteria. A grade 1 or mild was considered a noticeable adverse event, but one which does not interfere with normal daily activities; a grade 2 or moderate adverse event is one which is sufficient to interfere with normal daily activities; a grade 3 or severe adverse event is incapacitating and causes the patient to be unable to perform daily activities (hospitalization may or may not be required); a grade 4 or life-threatening adverse event poses an immediate risk of death as it occurs; and a grade 5 adverse event is fatal. If severe toxicity attributable to active viral replication had occurred, initiation of intravenous ribavirine therapy was recommended at 35 mg/kg divided into three doses given every 8 h (the first day) followed by 25 mg/kg divided into three daily doses and given every 8 h; no patients required ribavirine therapy.

Screening and pretreatment evaluation

CT scans, chest radiographs or ultrasound evaluations were completed within 21 days before treatment. All other examinations and procedures were completed within 14 days before the initial treatment with ONYX-015. The biopsies documenting carcinoma and evaluation of p53 status were taken before enrollment or treatment with ONYX-015. Screening and pretreatment evaluation included a signed IRB-approved informed consent form, CT-scan evaluation of the chest and/or liver/abdomen with tumor measurements, chest X-ray (PA/lateral), 12-lead electrocardiogram (ECG), complete medical history including secondary diagnosis, complete physical examination, medications within the last 2 months, previous anticancer treatments, Karnofsky performance status score determination. Screening laboratory assessment included hematological tests, including prothrombin time (PT), partial thromboplastin time (PTT), serum chemistry tests, neutralizing antibody to ONYX-015, ONYX-015 DNA in plasma, urinalysis, urine or serum pregnancy test, and a paraffin tumor block. Plasma samples were also archived.

Treatment evaluation on days 1, 8 and 15

For administration of ONYX-015, patients were treated at the Mary Crowley Medical Research Center of US Oncology located in the Sammons Cancer Center at Baylor University Medical Center, Dallas, TX, USA. Vital sign assessments were taken at baseline (45 min before ONYX-015 infusion ± 15 min). In addition, vital signs were monitored just before infusion, at the conclusion of the infusion and every 15 min thereafter for a total of 2 h. If vital signs had changed ±20% from baseline after 2 h, patients were to be retained, examined and had vital signs taken until they returned to baseline values.

Procedures during the ONYX-015 treatment period
Day 1 (±1 day) procedures included a brief physical examination directed to relevant signs, symptoms, vital signs and weight, hematological tests, serum chemistry tests including liver function, cytokine analysis (baseline and 6 h, ±1 h, following injection of the virus) for cycles 1 and 2 only. On day 2 (±1 day), cytokine analysis (24 h, ±3 h, following injection of the virus) was done on cycles 1 and 2. On day 3 (±1 day), serum liver function tests were repeated, plasma for PCR (polymerase chain reaction) and antibody titer (only cycles 1 and 2) was obtained, and cytokine analysis (48 h, ±3 h, following injection of the virus) was done in cycles 1 and 2 only. On day 4 (±1 day), cytokine analysis (48 h, ±3 h, following injection of the virus) was done in cycles 1 and 2. On day 5 (±1 day), serum liver function tests were repeated, and three or more biopsies of tumor and normal tissue (liver, pulmonary, skin) on cycle 1 only were done to assess for replicating ONYX-015. Cytokine analysis (96 h, ±3 h, following the first injection of each cycle of the virus) was also done in cycles 1 and 2. On days 8 to 15 (±2 days) of the second and third infusion of ONYX-015, hematological tests, serum chemistry tests including liver function, and physical examination were repeated. Changes in concomitant medications and reporting of adverse events were summarized. A plasma sample was archived (cycles 1 and 2 only). Neutralizing antibody to ONYX-015 (only cycles 1 and 2), plasma for PCR, ONYX-015 titer (only cycles 1 and 2), and cytokine analysis (168 h, ±3 h, following the first injection of each cycle the virus) were done in cycles 1 and 2.

Pharmacokinetic venous blood draws
All patients had pharmacokinetic blood draws taken on day 1 of cycles 1 and 2. Blood was drawn at the start of the ONYX-015 infusion and at 5, 10, 30, 60, 90, 120, 180 and 360 min. Pharmacokinetics were repeated for the first dose of cycle 2 at time 0 before ONYX-015 infusion and at 30, 90, 120 and 360 min. Samples were also drawn before ONYX-015 infusion on days 8 and 15.

Off study assessment
Patients who completed their final cycle of ONYX-015 treatment or came off study for other reasons were withdrawn from study and had the following assessments completed: baseline physical examination, Karnofsky performance status, labs for hematology and chemistry, urinalysis, adverse events, and concomitant medications.

Long-term follow-up
If patients went off study with an objective tumor response or stable disease (ie without tumor progression), an abdominal CT scan or ultrasound was taken every 6–8 weeks to follow for disease progression. All patients who went off study for any reason were followed for survival.

Cytokine analysis
Cytokines IL-6, IL-1α, IL-1β, TNF-α, IL-10, IL-12, and γ-IFN were assayed from patient serum. ELISA assays (R+D Quantikine kits; Minneapolis, MN, USA) were carried out to quantify cytokine levels. Post-treatment cytokine levels were compared with baseline levels harvested before infusion 1 of cycle 1.

Patient peripheral blood was collected by venipuncture into Vacutainer tubes without anticoagulant. Serum samples were extracted from clotted samples and stored at –80°C. Multiple serum samples collected over each patient's treatment course were analyzed simultaneously (in triplicate), using cytokine-specific immunoassay reagents according to the manufacturer's protocols. The colorimetric reaction was quantified as a function of OD absorbance at 540 nm (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA). Cytokine concentration was calculated according to a reference standard curve generated with four parameter logistic (4-PL) curve fit and OD values of known, graded concentrations of recombinant cytokine. The lowest concentration of each cytokine's standard curve consistently yielded an OD values that are ≥3-fold higher than the $OD_{background}$ (no recombinant cytokine or serum added), and are considered to be the minimal detectable level (IL-6: 3.1 pg/ml; IL-1α/IL-1β: 3.9 pg/ml; TNF-α: 15.6 pg/ml; IL-10: 7.8 pg/ml; IL-2: 31.2 pg/ml; IFN-γ: 15.6 pg/ml). Post-treatment cytokine levels were compared with baseline levels harvested before infusion 1 of cycle 1 by the formula: % increase over baseline = $100\% \times (C_T - C_0)/C_0$; where C_T and C_0 represent the mean calculated cytokine concentration at time-point T, and before treatment, respectively.

Statistical analysis
The study included seven treatment cohorts, with one to six patients in each cohort. The data for each cohort were summarized with descriptive statistics, frequency tabulations, graphs, and data listings as appropriate. The data from the treatment cohorts were combined for selected data displays. Parametric or non-parametric methods were used to compare dose groups, as appropriate.

P53 gene sequencing
Pretreatment tumor biopsies were taken for p53 sequencing from the recurrent tumor mass that was to be injected (sequencing performed at the John T Mallams Laboratory, supported by the Mary C Crowley Foundation). DNA was extracted directly from sections of formalin-fixed paraffin-embedded needle biopsies. If necessary, tumor cells were micro-dissected from sections of paraffin-embedded tissue. DNA was isolated from tumor cells using phenol/chloroform extraction and ethanol precipitation, and the p53 gene was amplified in several fragments by PCR. The amplicons were purified by gel electrophoresis and analyzed using either the DNA Sequencing Kit (Boehringer Mannheim, Indianapolis, IN, USA), or the ABI Model 310 automated sequencer. Accurate reading of the sequencing gels and the sequencer printout was confirmed by National Biosciences (Plymouth, MN, USA).

Quantitative PCR of ONYX-015
To quantify the amount of ONYX-015 virus genome in human plasma, viral DNA was extracted from patient samples, standard and controls using a QIAamp DNA mini kit (Valencia, CA, USA) then quantified by real-time PCR using assay-specific primer and probe as described below. All tissue samples were analyzed independently by Althea Technologies (San Diego, CA, USA). In order to monitor recovery during extraction, patient samples were spiked with exogenous DNA. As PCR tumor tissue analysis had not been previously studied with other

patient tumor samples, a TaqMan-based assay was developed using a primer based on a 92 nucleotide (nt) sequence from nt 2453 to nt 2544. This amplicon overlaps the E1B region deletion and includes the 8bp Puc-derived linker insert. The central region of the TaqMan probe is homologous to the linker insert, making the probe specific to ONYX-015.

For quantification, a standard curve was constructed by assaying serial dilutions of ONYX-015 virus ranging from 2×10^9–1.05×10^4 vp/ml. Negative controls consist of a plasma control without virus and a type D adenovirus wild-type control. All samples were done with 1 μg of human placental DNA as background. The correlation coefficient of the standard curve was 1.00. The presence of PCR inhibitors in any sample was detected by the results of an independent PCR reaction using a primer specific for 18s rDNA.

Using this assay, the lower limits of detection of ONYX-015 particles in plasma was 1.05×10^4 particles/ml with a lower limit of quantitation of 4.2×10^4 particles/ml of plasma. In tissue, the lower limit of detection was 13 copies of ONYX-015 genome/μg with a quantitation limit of 23 copies/μg.

Histochemical assessment of adenoviral capsid antigen

Immunohistochemical studies for adenovirus were performed using a mouse monoclonal antibody against adenoviral capsid proteins (adenovirus clone 20/11 and 2/6; Cell Marrque, Austin, TX, USA). Formalin-fixed, paraffin-embedded sections were studied using an automated Ventana 320ES system (Tucson, AZ, USA) and a 1:10 dilution of the primary antibody. For detection, an avidin–biotin complex secondary antibody technique was used. Adequate negative and positive controls were included.

Determination of ONYX-015 neutralizing antibody titers

Patient and control samples were incubated at 55°C for 30 min to inactivate complement. Clinical plasma samples previously determined to produce high, midrange and negative titers were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titer prequalified to produce 15–20 plaques per well of a 12-well dish in DMEM growth medium. The patient's samples and controls were inoculated for 1 h at room temperature, and applied to 70–80% confluent JH393 cells in 12-well dishes. After 2 h of incubation at 37°C, 5% CO_2 plasma-virus mix was removed and 2 ml of 1.5% agarose in DMEM was added to each well. Plates were read on day 7 after inoculation by counting the number of p.f.u.s per well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody.

Acknowledgements

We wish to thank Ana Petrovich for manuscript preparation, Sherry Toney for extensive time and effort in coordinating study samples and results as well as editorial proofing of the manuscript, and George Smith and Carrie LeDuc from Althea for providing extensive assistance in determining ONYX-015 DNA analysis.

References

1 Kozarsky K, Grossman M, Wilson JM. Adenovirus-mediated correction of the genetic defect in hepatocytes from patients with familial hypercholesterolemia. *Somat Cell Mol Genet* 1993; **19**: 449–458.
2 Roth JA, Cristiano RJ. Gene therapy for cancer: what have we done and where are we going? *J Natl Cancer Inst* 1997; **89**: 21–39.
3 Martuza RL *et al*. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* 1991; **252**: 854–856.
4 Kirn D. Replication-selective micro-organisms: fighting cancer with targeted germ warfare. *J Clin Invest* 2000; **105**: 836–838.
5 Heise C, Kirn D. Replication-selective adenoviruses for cancer. *J Clin Invest* 2000; **105**: 847–851.
6 Hawkins L, Nye J, Castro D, Kirn D. Replicating adenoviral gene therapy. *Proc AACR* 1999; **40**: 476.
7 Freytag SO *et al*. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther* 1998; **9**: 1323–1333.
8 Miller D. Gene therapy on trial. *Science* 2000; **288**: 951–957.
9 Lechner MS *et al*. Human papilloma virus E6 proteins bind p53 *in vivo* and abrogate p53-mediated repression of transcription. *EMBO J* 1992; **1**: 3045–3052.
10 Gannon JV, Lane DP. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature* 1987; **329**: 456–458.
11 Bischoff JR *et al*. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 1996; **274**: 373–376.
12 Harris C, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 1993; **329**: 1318–1326.
13 Heise C *et al*. ONXY-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nature Med* 1997; **3**: 639–645.
14 Goodrum FD, Ornelles DA. The early region 1B 55-kilodalton oncoprotein of adenovirus relieved growth restrictions imposed on viral replication by the cell cycle. *J Virol* 1997; **71**: 548–561.
15 Turnell AS, Grand RJ, Gallimore PH. The replicative capacities of large E1B-null group A and group C adenoviruses are independent of host cell p53 status. *J Virol* 1999; **73**: 2074–2083.
16 Rothmann T *et al*. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J Virol* 1998; **72**: 9470–9478.
17 Fujiwara T *et al*. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type p53 gene. *Cancer Res* 1994; **54**: 2287–2291.
18 Heise C *et al*. Intravenous administration of ONYX-015, a replication-selective adenovirus, induces antitumoral efficacy. *Cancer Res* 1999; **59**: 2623–2628.
19 You L, Yang C-T, Jablons DM. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res* 2000; **60**: 1009–1013.
20 Khuri F *et al*. A controlled trial of intratumoral ONYX-015, a selectively replicating adenovirus, in combination with cisplatin and 5-FU in patients with recurrent head and neck cancer. *Nature Med* 2000; **6**: 879–885.
21 Gordon MS *et al*. A phase I trial of recombinant human IL-6 in patients with myelodysplastic syndromes and thrombocytopenia. *Blood* 1995; **85**: 3066–3076.
22 Nemunaitis J *et al*. Human marrow stromal cells: response to IL-6 and control of IL-6 expression. *Blood* 1989; **74**: 1929–1935.
23 O'Neil WK. Toxicological comparison of E2A-deleted and first generation adenoviral vectors expressing alpha-1-antitrypsin after systemic delivery. *Hum Gene Ther* 1998; **9**: 1587–1598.
24 Yang Y, Xiang H, Ertl J, Wilson JM. Upregulation of class I major histocompatibility complex antigens by γ-IFN is necessary for T-cell mediated elimination of recombinant adenovirus-injected hepatocytes *in vivo*. *Proc Natl Acad Sci USA* 1995; **92**: 7257–7261.

25 Yang Y, Li Q, Erth HC, Wilson JM. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenovirus. *J Virol* 1995; **69**: 2004–2015.
26 Yang Y, Greenough K, Wilson JM. Transient immune blockade prevents formation of neutralizing antibody to recombinant adenovirus and allows repeated gene transfer to mouse liver. *Gene Therapy* 1996; **3**: 412–420.
27 Ganly I *et al*. A phase I study of ONYX-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res* 2000; **6**: 798–806.
28 Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. *Hum Gene Ther* 1997; **8**: 37–44.
29 Kass-Eisler A *et al*. Circumventing the immune response to adenovirus-mediated gene therapy. *Gene Therapy* 1996; **3**: 154–162.
30 Huard J *et al*. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Therapy* 1995; **2**: 107–115.
31 Wolff G *et al*. Enhancement of *in vivo* adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. *J Virol* 1997; **71**: 624–629.
32 Biewenga J *et al*. Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund's adjuvant. *Cell Tissue Res* 1995; **280**: 189–196.
33 McCuskey RS, McCuskey PA, Urbaschek R, Urbaschek B. Kupffer cell function in host defense. *Rev Infect Dis* 1999; **9**: S616–S619.
34 Huitinga I *et al*. Macrophages in T-cell line-mediated demyelination and chronic relapsing experimental autoimmune encephalomyelitis in Lewis rats. *Clin Exp Immunol* 1995; **10**: 344–351.
35 Laman JD, Kors N, van Rooijen N, Claassen E. Mechanism of follicular trapping localization of immune complexes and cell remnants after elimination and repopulation of different spleen cell populations. *Immunology* 1990; **71**: 57–62.
36 Pinto AJ, Stewart D, vanRooijen N, Morahan PS. Selective depletion of liver and splenic macrophages using lyposomes encapsulating the drug dichloromethylene diphosphonate: effects of antimicrobial resistance. *J Leukoc Biol* 1991; **49**: 579–586.
37 Qian Q, Jutila A, van Rooijen N, Cutler JE. Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. *J Immunol* 1994; **152**: 5000–5008.
38 Tscaikowsky D, Brain JD. Effects of liposome-encapsulated dichloromethylene diphosphonate on macrophage function and endotoxin-induced mortality. *Biochem Biophys Acta* 1994; **1222**: 323–330.
39 van Rooijen N. The liposome-mediated macrophage 'suicide' technique. *J Immunol Meth* 1989; **124**: 1–6.
40 van Rooijen N, Kors N, Kraal G. Macrophage subset repopulation in the spleen differential kinetics after liposome-mediated elimination. *J Leukoc Biol* 1989; **45**: 97–104.
41 van Rooijen N, Kors N, van der Ende M, Kijkstra CD. Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphone. *Cell Tissue Res* 1990; **260**: 215–222.
42 van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action preparation of lyposomes and application. *J Immunol Meth* 1994; **174**: 83–93.
43 van Rooijen N, Sanders A. Kupffer cell depletion by liposome-delivered drugs: comparative activity of intracellular clodronate propamidine and ethylenediaminetetraacetic acid. *Hepatology* 1996; **23**: 1239–1243.
44 Vreden SG *et al*. Kupffer cell elimination enhances development of liver schizonts of Plasmodium berghei in rats. *Infect Immunol* 1993; **61**: 1936–1939.
45 Li E, Stupack D, Bokoch GM, Nemerow GR. Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol* 1998; **72**: 8806–8812.
46 Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins $\alpha_v\beta_5$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**: 309–319.
47 Chardonnet Y, Dales S. Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. *Virology* 1970; **40**: 462–477.
48 Patterson S, Russell WC. Ultrastructural and immunofluorescence studies of early events in adenovirus–HeLa cell interaction. *J Gen Virol* 1983; **64**: 1091–1099.
49 Li E *et al*. Adenovirus endocytosis via α_v integrins requires phosphoinositide-3-OH-kinase. *J Virol* 1998; **72**: 2055–2061.
50 Bokoch GM *et al*. Rac GTPase interacts specifically with phosphatidylinositol 3-kinase. *Biochem J* 1996; **315**: 775–779.
51 Rodriguez-Viciana P *et al*. Phosphotidylinositol-3-OH kinase as a direct target of *ras*. *Nature* 1994; **370**: 527–532.
52 Tolias KF, Cantley L, Carpenter CL. Rho family GTPases bind to phosphoinositide kinase. *J Biol Chem* 1995; **270**: 17656–17659.
53 Zhang Y, Bagrodia S, Cerione RA. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. *J Biol Chem* 1994; **269**: 18727–18730.
54 Li Q *et al*. Assessment of recombinant adenoviral vectors for hepatic gene therapy. *Hum Gene Ther* 1993; **4**: 403–409.
55 Kjellen L, Pereira HG. Role of adenovirus antigens in the induction of virus neutralizing antibody. *J Gen Virol* 1968; **2**: 177–185.
56 Wohlfart C. Neutralization of adenoviruses: kinetics, stoichiometry and mechanisms. *J Virol* 1988; **62**: 2321–2328.
57 Greber UF, Willetts M, Webster P, Helenius A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 1993; **75**: 477–486.
58 Bai M, Campisi L, Freimuth P. Vitronectin receptor antibodies inhibit infection of HeLa and A549 cells by adenovirus type 12 but not by adenovirus type 2. *J Virol* 1994; **68**: 5925–5932.
59 Goldman MJ, Wilson JM. Expression of $\alpha_v\beta_5$ integrin is necessary for efficient adenovirus-mediated gene transfer in the human airway. *J Virol* 1995; **69**: 5951–5958.
60 Wohlfart C, Svensson UK, Everitt E. Interaction between HeLa cells and adenovirus type 2 virions neutralized by different antisera. *J Virol* 1985; **56**: 896–903.
61 Shenk T. Adenoviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM (eds). *Fields Virology*. Lippincott-Raven: Philadelphia, 1996, 2111.
62 Yang Y, Ertl HC, Wilson JM. MHC class I-restricted cytotoxic T-lymphocytes to viral antigens destroy hepatocytes in mice-infected with E1-deleted recombinant adenoviruses. *Immunity* 1994; **1**: 433–442.
63 Barr D *et al*. Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes *in vivo*: comparisons between immunocompetent and immunodeficient inbred strains. *Gene Therapy* 1995; **2**: 151–155.
64 Dai Y *et al*. Cellular and humoral immune response to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc Natl Acad Sci USA* 1995; **92**: 1401–1405.
65 Zsengeller ZK *et al*. Persistence of replication-deficient adenovirus-mediated gene transfer in lung of immune-deficient (nu/nu) mice. *Hum Gene Ther* 1995; **6**: 457–467.
66 Kass-Eisler A *et al*. The impact of development stage, route of administration and the immune system on adenovirus-mediated gene transfer. *Gene Therapy* 1994; **1**: 395–402.
67 Kay MA *et al*. Long-term hepatic adenovirus-mediated gene expression in mice following CTLA41g administration. *Nat Genet* 1995; **11**: 191–197.
68 Crystal RG *et al*. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat Genet* 1994; **8**: 42–51.
69 Yang Y, Trinchieri G, Wilson JM. Recombinant IL-12 prevents formation of blocking IfA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. *Nature Med* 1995; **1**: 890–893.

70 Kolls JK *et al*. Use of transient CD4 lymphocyte depletion to prolong transgene expression of E1-deleted adenoviral vectors. *Hum Gene Ther* 1996; **7**: 489–497.
71 Sawchuk SJ *et al*. Anti-T-cell receptor monoclonal antibody prolongs transgene expression following adenovirus-mediated *in vivo* gene transfer to mouse synovium. *Hum Gene Ther* 1996; **7**: 499–506.
72 Bouvet M *et al*. Suppression of the immune response to an adenovirus vector and enhancement of intratumoral transgene expression by low dose etoposide. *Gene Therapy* 1998; **5**: 189–195.
73 Fang B. Gene therapy for hemophilia B: host immunosuppression prolongs the therapeutic effect of adenovirus-mediated factor IX expression. *Hum Gene Ther* 1995; **6**: 1039–1044.
74 Jooss K, Ertl HC, Wilson JM. Cytotoxic T-lymphocyte target proteins and their major histocompatibility complex class I restriction in response to adenovirus vectors delivered to mouse liver. *J Virol* 1998; **72**: 2945–2954.
75 Engelhardt JF, Ye X, Doranz B, Wilson JM. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci USA* 1994; **91**: 6196–6200.
76 Vilquin JT *et al*. FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. *Hum Gene Ther* 1995; **6**: 1391–1401.
77 Lochmuller H *et al*. Immunosuppression by FK506 markedly prolongs expression of adenovirus-delivered transgene in skeletal muscles of adult dystrophic (mdx) mice. *Biochem Biophys Res Commun* 1995; **213**: 569–574.
78 Wold WS, Hermiston TW, Tollefson AE. Adenovirus proteins that subvent host defenses. *Trends Microbiol* 1994; **2**: 437–443.
79 Lee MG, Abina MA, Haddada H, Perricaudet M. The constitutive expression of the immunomodulatory gp 19k protein in E1-, E3 adenoviral vectors strongly reduces the host cytotoxic T-cell response against the vector. *Gene Therapy* 1995; **2**: 256–262.
80 Wold WS, Tollefson AE, Hermiston TW. E3 transcription unit of adenovirus. *Curr Top Microbiol Immunol* 1995; **199**: 237–274.
81 Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci USA* 1994; **91**: 8802–8806.
82 Ilan Y *et al*. Insertion of the adenoviral E3 region into a recombinant viral vector prevents antiviral humoral and cellular immune responses and permits long-term gene expression. *Proc Natl Acad Sci USA* 1997; **94**: 2587–2592.
83 Horwitz MS, Tufariello J, Grunhaus A. Model system for studying the effects of adenovirus E3 gene on virulence *in vivo*. *Curr Top Microbiol Immunol* 1995; **199**: 195–211.
84 Rawle FC, Tollefson AE, Wold WS, Gooding LR. Mouse anti-adenovirus cytotoxic T-lymphocytes. Inhibition of lysis by E3 gp19K but not E1 14.7K. *J Immunol* 1989; **143**: 2031–2037.
85 Wold WS, Gooding LR. Region E3 of adenovirus: a cassette of genes involved in host immunosurveillance and virus-cell interactions. *Virology* 1991; **184**: 1–8.
86 Feuerbach D, Burgert HG. Novel proteins associated with MHC class I antigens in cells expressing the adenovirus protein E3/19K. *EMBO J* 1993; **12**: 3153–3161.
87 Beier DC *et al*. Association of human class I MHC alleles with the adenovirus E8/19K protein. *J Immunol* 1994; **152**: 3862–3872.
88 Kaplan JM *et al*. Characterization of factors involved in modulating persistence of transgene expression from recombinant adenovirus in the mouse lung. *Hum Gene Ther* 1997; **8**: 45–56.
89 Medina DJ *et al*. Adenovirus-mediated cytotoxicity of chronic lymphocytic leukemia cells. *Blood* 1999; **94**: 3499–3508.
90 Rodriguez R *et al*. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* 1997; **57**: 2559–2563.
91 Barker DD, Berk AJ. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral injection and DNA transfection. *Virology* 1987; **156**: 107–121.